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RESEARCH PAPER

Induction of secondary dormancy by hypoxia in barley grains and its hormonal regulation

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Abstract

In barley, primary dormant grains did not germinate at 30 °C in air and at 15 °C in an atmosphere containing less than 10% O_2, while they germinated easily at 15 °C in air. O_2 tension in embryos measured with microsensors was 15.8% at 15 °C but only 0.3% at 30 °C. Incubation of grains at 30 °C is known to induce secondary dormancy in barley, and it was shown here that secondary dormancy was also induced by a 3 d treatment in O_2 tensions lower than 10% at 15 °C. After such treatments, the grains lost their ability to germinate subsequently at 15 °C in air. During seed treatment in 5% O_2, embryo abscisic acid (ABA) content decreased more slowly than in air and was not altered after transfer into air. Hypoxia did not alter the expression of ABA metabolism genes after 1 d, and induction of *HvNCED2* occurred only after 3 d in hypoxia. Embryo sensitivity to ABA was similar in both primary and hypoxia-induced secondary dormant grains. Gibberellic acid (GA) metabolism genes were highly regulated and regulated earlier by the hypoxia treatment, with major changes in *HvGA2ox3*, *HvGA3ox2* and *HvGA20ox1* expression after 1 d, resulting in reduced GA signalling. Although a high temperature has an indirect effect on O_2 availability, the data showed that it did not affect expression of prolyl-4-hydroxylases and that induction of secondary dormancy by hypoxia at 15 °C or by high temperature in air involved separate signalling pathways. Induction by hypoxia at 15 °C appears to be more regulated by GA and less by ABA than the induction by high temperature.

Key words: abscisic acid, germination, gibberellins, hypoxia, seed, secondary dormancy.

Introduction

Dormancy is defined as the inability to germinate in apparently favourable conditions (Bewley, 1997). This process is labile, as dormant seeds generally become non-dormant after dry storage (after-ripening) or after imbibition at cold temperatures (stratification). Seed dormancy inhibits untimely germination on the mother plant or just after seed release. It is an adaptation that allows germination and seedling establishment in more favourable seasons. Dormancy can be imposed either by the embryo or the seed coat or both according to the species. Indeed, the alleviation of dormancy represents a widening of the conditions in which the seeds can germinate, with dormant seeds only able to germinate in restricted conditions. Two types of dormancy are distinguished in seeds: primary dormancy is induced during seed development, while secondary dormancy is induced in the mature seeds by unfavourable conditions (Hilhorst, 2007). Dormancy is thus regulated by environmental factors such as temperature, water content, light, and O_2, and by internal factors such as phytohormones. In soil seed banks, according to the environmental conditions, dormancy cycling will be observed with alleviation of primary dormancy and induction of secondary dormancy over time (Baskin and Baskin, 1998; Footitt *et al.*, 2011). The presence of dormant seed banks in soils provides an opportunity for germination to occur over several seasons, thus maximizing the chance of population establishment (Baskin and Baskin, 1998; Gubler *et al.*, 2005).

The barley caryopses, like most cereal grains, are dormant at harvest as they do not germinate at high temperatures

(above 25 °C), but they germinate readily at lower temperatures (5–20 °C) (Corbineau and Côme, 1996). As O_2 solubility decreases with increased temperature, the temperature effects could also be related to O_2 availability in the embryo. In cereal grains such as barley, O_2 availability is restricted largely by the presence of adhering glumellae, which can trap O_2 by oxidation of phenolic compounds by polyphenoloxidases (Lenoir *et al*., 1986). A similar process is assumed for the seed coat-imposed dormancy of *Arabidopsis* and wheat (Flintham, 1993; Debeaujon *et al*., 2000; 2007; Himi *et al*., 2002). Characterization of the sensitivity to O_2 of seed germination, using a population-based threshold model in 16 species, shows that the O_2 percentage required for 50% germination ranges from 21 to as low as 0.005%, depending on the species, the temperature, and the dormancy depth (Bradford *et al*., 2007). Analysis of barley using this method has shown that dormancy and germination are regulated by a combination of physical (O_2 diffusion through the hull) and physiological [abscisic acid (ABA) and gibberellin acid (GA) sensitivities] factors (Bradford *et al*., 2008). This study highlighted the fact that limitation of O_2 availability to the embryo is apparently a key regulator of germination, in particular via its effects on ABA sensitivity of the embryo, in agreement with data published by Benech-Arnold *et al*. (2006), which clearly demonstrated that hypoxia resulted in an increase in embryo sensitivity to ABA. To date, most studies on cereal dormancy have been carried out on primary dormant seeds, and processes regulating secondary dormancy induction remain poorly understood. The incubation of mature cereal grains at high temperatures has been shown to induce secondary dormancy (Corbineau *et al*., 1993; Leymarie *et al*., 2008; Hoang *et al*., 2012) and, given the role of O_2 supply in primary dormancy, the effect of hypoxia on the induction of secondary dormancy could be important. Hypoxia has been shown to induce secondary dormancy in seeds of apple (Côme and Tissaoui, 1968), *Xanthium pennsylvanicum* (Esashi *et al*., 1978), *Viola* (Lonchamp and Gora, 1979) and rape (Pekrun *et al*., 1997). A similar effect has been observed in anoxia for *Avena fatua* (Symons *et al*., 1986) and *Datura stramonium* (Benvenuti and Macchia, 1995).

The effects of hypoxia have been studied mainly in plants roots in the flooding context (Bailey-Serres and Voesenek, 2008), but the mechanism of O_2 sensing in plants is not well known. In mammals, a central regulator of hypoxic gene expression is the hypoxia-induced factor (HIF) transcription factor, regulated post-translationally by prolyl-4-hydroxylase (P4H) (reviewed by Kaelin and Ratcliffe, 2008). In yeast, Ofd1, a P4H-like protein, is considered to be an O_2 sensor and regulates Sre1N degradation by the proteasome (Hughes and Espenshade, 2008; Hughes *et al*., 2009). In plants, no protein or gene presenting homology with HIF or Ofd1 could be found by sequence analyses (Hughes and Espenshade, 2008), but several P4H were shown to be involved in the hypoxia response (Vlad *et al*., 2007; Asif *et al*., 2009), suggesting similar pathways exist in plants. Moreover, P4H genes are subjected to alternative splicing in maize seedlings under waterlogging conditions (Zou *et al*., 2011), mechanism already known to regulate P4H genes in human cells under hypoxia (Hirsilä *et al*., 2003). Recently, Gibbs *et al*. (2011) and Licausi *et al*. (2011) showed that the N-end rule pathway of targeted proteolysis acts as a homeostatic sensor of severe low O_2 in *Arabidopsis*, through its regulation of group VII ethylene-response factor transcription factors (Bailey-Serres *et al*., 2012).

As high temperature induces secondary dormancy in barley (Leymarie *et al*., 2008) in relation to water content (Hoang *et al*., 2012), we determined whether hypoxia could induce secondary dormancy in this species and whether the mechanisms were common or specific to the inductive condition. Special attention was paid to the ABA and GA metabolisms known to be key regulators of primary and secondary dormancies in barley (Chono *et al*., 2006; Millar *et al*., 2006; Leymarie *et al*., 2008; Hoang *et al*., 2012). In addition, we investigated the expression of genes encoding P4Hs putatively regulated by hypoxia at 30 °C and in hypoxia. Our results showed that, in contrast to primary dormancy, which is regulated in part through changes in ABA catabolism and synthesis (Chono *et al*., 2006; Millar *et al*., 2006), hypoxia-triggered secondary dormancy was induced solely through increased expression of ABA synthesis genes in the embryo. Moreover, GA metabolism genes were highly regulated and were regulated earlier by the hypoxia treatment, leading to the conclusion that induction of the secondary dormancy by high temperature in air or by hypoxia at 15 °C involves separate signalling pathways.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L., cv. Pewter) grains, harvested in July 2008, and kindly provided by the 'Coopérative agricole de la Beauce et du Perche' (28310 Toury, France) were used throughout this study. Experiments were carried out on dormant grains, which were stored at –20 °C from harvest until the experiments began in order to maintain their primary dormancy (Lenoir *et al*., 1986).

Germination assays

Germination assays were performed at 15 or 30 °C in darkness, in four replicates of 50 grains or 25 embryos placed in 9 cm diameter Petri dishes on a layer of cotton wool soaked with water or chemical solutions. Isolated embryos were excised with a scalpel blade from grains in a dry state or after treatment for secondary dormancy induction and incubated in Petri dishes as for the grains above. ABA, GA_3 and fluridone were purchased from Sigma. Atmospheric O_2 content was modulated using the procedure of Côme and Tissaoui (1968); gas mixtures containing 1–21% O_2 were obtained through capillary tubes connected to sources of compressed air and N_2. The gaseous atmospheres were passed continuously through germination boxes at a constant flow rate (4.0 l h^{-1}). The O_2 tension was measured daily using a Servomex analyser (Type 570A, Servomex, The Netherlands).

Germination was considered when the coleorhiza had protruded through the seed-covering structures. Germination counts were conducted regularly for 7 d. The results presented correspond to the mean of the germination percentages obtained for four replicates ±standard deviation (SD). The viability of the non-germinated grains was checked with a tetrazolium test. For this purpose, longitudinally cut grains were incubated in a 1% 2,3,5-triphenyltetrazolium chloride salt solution for 1–2 h at 25 °C in darkness (Lehner *et al*., 2008).

Embryo ABA content measurement

About 30–40 isolated embryos were frozen immediately in liquid N_2, lyophilized, and weighed. The ABA content was determined by an HPLC ELISA method (Julliard *et al*., 1994), as described by Bahin *et al*. (2011). The results presented correspond to the mean of five biological replicates ± SD.

Determination of embryo O_2 content

Embryo O_2 content was determined using a fibre-optic O_2 microsensor, microX TX3 (Presens, Regensburg, Germany) with flat-broken sensor tips (140 µm diameter). The microsensor was calibrated with ambient air (21% O_2) and O_2-deprived air (1% Na_2SO_3 solution) at the same temperature. After fixation on a micromanipulator, a small hole was created in the embryos using a needle and the sensor tip was inserted in the hole. To prevent diffusion of O_2, silicone was applied around the protective needle on the grain surface. The glass fibre was then driven out of the protective needle and the O_2 content was determined as a percentage. For each point, 15 grains were analysed.

RNA extraction

Isolated embryos were frozen immediately in liquid N_2 and stored at –80 °C. For each extraction, 25 embryos were ground in liquid N_2 using a laboratory mixer (Retsch) mill with stainless steel balls, and total RNA was extracted according to the method of Verwoerd *et al*. (1989) using a hot phenol procedure. The RNA extract was analysed using a spectrophotometer (Nanovue; GE Healthcare) to measure absorbance at 260 nm (A_{260}) to determine the RNA concentration. RNA quality was evaluated by determining the A_{260}/A_{280} ratio and was also checked by agarose gel electrophoresis.

Real-time quantitative RT-PCR

Real-time RT-PCR was performed as described by Hoang *et al*. (2012) from 2 µg of total RNA. The sequences of the primers used are presented in Table S1 (at *JXB* online). Relative expression was calculated according to the method of Hellemans *et al*. (2007) with at least three of the reference genes *HvActin*, *Hv18S*, *HvEF1α* and *HvMub1*. An arbitrary value of 100 was assigned to the dry dormant grain samples, which were used as the control sample for normalization (Hellemans *et al*., 2007).

Statistical analyses

Data from the real-time RT-PCR were analysed with StatBox 6.40 software (Grimmer Logiciel, Paris, France).

Results

Sensitivity to hypoxia

Primary dormant barley grains germinated readily at 15 °C in air, but germination was inhibited by an O_2 tension of less than 10% O_2 and was completely abolished below 5% O_2, and a tetrazolium test demonstrated that non-germinated grains in hypoxia (1–10% O_2) remained viable (Table S2 at *JXB* online). Under the seed-covering structures, the embryo O_2 content, measured with microsensors, was dramatically different at 15 and 30 °C, being around 0.3% at 30 °C and closer to atmospheric value (15.8%) at 15 °C (Table 1). However, the expression of both P4H (*HvP4H1* and *HvP4H2*) genes was not significantly altered by high temperature or hypoxia (Fig. S1 at *JXB* online).

Induction of secondary dormancy by hypoxia

To determine whether hypoxia could induce of secondary dormancy, grains were placed for 3 d at various O_2 tensions at 15 °C and 30 °C, and then transferred to air at 15 °C. It is known that a 30 °C treatment in air can also induce secondary dormancy (Leymarie *et al*., 2008; Hoang *et al*., 2012). This induction of secondary dormancy by high temperature was not altered by hypoxia (Fig. 1, open circles). A pre-treatment at 15 °C in atmospheres containing less than 10% O_2 (conditions that inhibited the germination of primary dormant grains, Table S2) inhibited the subsequent germination percentage in air at 15 °C. Only 40% of grains pre-treated in 1–5% O_2 at 15 °C germinated after transfer to air (Fig. 1, black circles). The non-germinated grains were alive, as checked by a tetrazolium test of viability (data not shown). Thus, secondary dormancy was clearly induced by hypoxia at 15 °C in barley grains.

Embryo ABA content and sensitivity

The ABA content was determined in embryos during the induction of secondary dormancy after 1 and 3 d in 5% O_2 at 15 °C and when the secondary dormancy was expressed, i.e. 1 d after transfer at 15 °C in air. After 1 d in hypoxia, the ABA content remained at a similar level to that observed in dry grains [around 2.5 pmol mg^{-1} of dry weight (DW)], while in air this level decreased to 1.4 pmol mg^{-1} DW (Fig. 2A). Embryo ABA content decreased slowly after 3 d in hypoxia and after the transfer into air, being similar after the transfer to that observed in primary dormant grains placed at 15 °C in air (Fig. 2A). Fluridone, an inhibitor of ABA synthesis, had no significant effect on germination when applied during the inductive treatment or after the transfer to air (Table 2). It did not also affect the ABA content after 3 d at 15 °C and 5% O_2 (1.87 ± 0.29 pmol mg^{-1} DW; data not shown), which agrees with the absence of secondary induction. Fig. 2B shows the germination of embryos isolated from primary and secondary dormant grains placed at 30 °C in the presence of ABA at concentrations ranging from 0 to 1 mM. The responsiveness to ABA was similar for both types of embryo.

Expression of ABA metabolism genes

The change in embryo ABA content observed during induction and expression of secondary dormancy could be related to transcriptional regulation of the main genes known to

Table 1. Oxygen tension (%) at the embryo level determined with an oxygen microsensor. The sensor tip was placed in the embryo in the whole grain. Results are shown as the mean of 15 replicates ± SD.

Incubation conditions	O_2 (%)
30 °C, 1 d	0.32 ± 0.22
30 °C, 3 d	0.32 ± 0.17
15 °C, 1 d	15.77 ± 2.15

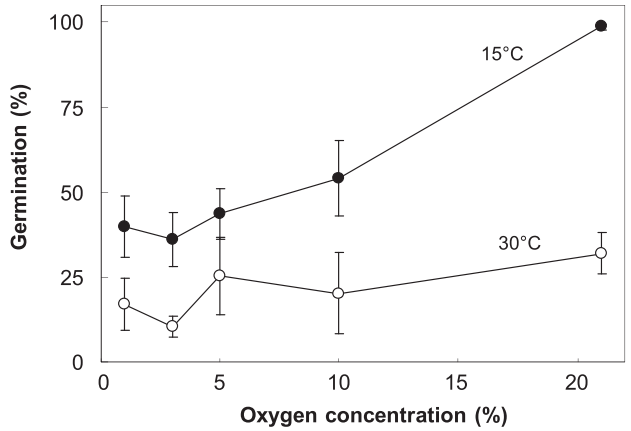

Fig. 1. Induction of secondary dormancy in whole grains by hypoxia. Primary dormant grains were incubated in darkness for 3 d in 1, 3, 5, 10, and 21% O_2 at 15 °C (filled circles) or 30 °C (open circles) and transferred to air at 15 °C. The data presented correspond to the germination percentage at 7 d after the treatment. Results are given as means of four replicates ± SD.

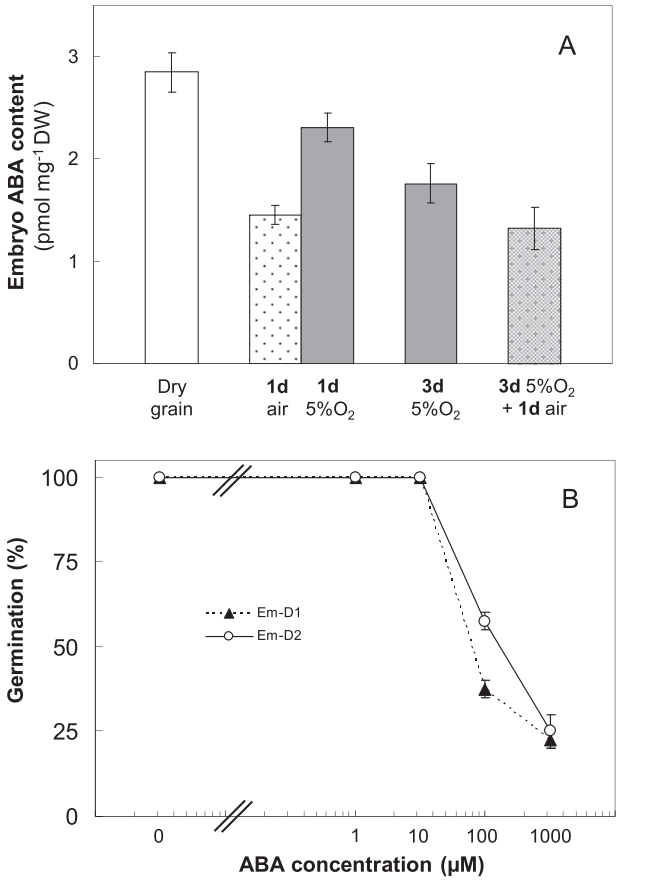

Fig. 2. Embryo ABA content and sensitivity. (A) ABA content (pmol mg^{-1} DW) of embryos isolated from primary dormant dry grains (open bar), grains placed for 1 d at 15 °C in air (dotted bar) and at 1 and 3 d at 15 °C in hypoxia (5% O_2) (grey bars), and from secondary dormant grains after transfer to air for 1 d (dotted grey bar). Results are given as means of five replicates ±SD. (B) Sensitivity to ABA of embryos from primary dormant grains (Em-D1, filled triangles) or from secondary dormant grains (Em-D2, open circles) at 30 °C (germination after 7 d). The secondary dormant grains were obtained from primary dormant grains incubated for 3 d in 5% O_2 at 15 °C. Results are given as means of four replicates ± SD.

regulate ABA content in barley: *HvABA8'OH1* for ABA catabolism, and *HvNCED1* and *HvNCED2* for ABA synthesis. *HvABA8'OH1* was expressed about fourfold more after 1 d of imbibition at 15 °C either in air or in hypoxia. However, after 3 d in hypoxia, i.e. in secondary dormant grains, *HvABA8'OH1* expression was reduced threefold and decreased even after the transfer to air (Fig. 3, open bars). The expression of *HvNCED* genes was not altered after 1 d of imbibition at 15 °C in air, and decreased a little after 1 d in 5% O_2, but after 3 d in 5% O_2, there was a large increase in the expression of *HvNCED2* that remained high after transfer (Fig. 3, grey bars).

Expression of GA metabolism and signalling genes

As GAs are difficult to measure, expression of several genes involved in GA inactivation (*HvGA2ox1*, *HvGA2ox3*, and *HvGA2ox5*), synthesis (*HvGA3ox2*, *HvGA20ox1*, and *HvGA20ox3*), or response (*HvExpA11*) was analysed in embryos during induction and expression of secondary dormancy (Figs 4 and S2 at *JXB* online). The main changes were observed for *HvGA2ox3* and *HvGA3ox2* (Fig. 4A, B). Indeed, incubation at 15 °C in air for 24 h, which allows germination, was associated with low expression of the *HvGA2ox3* gene and high expression of *HvGA3ox2*. Hypoxia treatment for 1 d resulted in a large increase in *HvGA2ox3* (64-fold compared with grains imbibed for 1 d in air) and reduced expression of *HvGA3ox2* (16-fold less compared with grains imbibed for 1 d in air). After 3 d in hypoxia, the high expression of *HvGA2ox3* was maintained, but *HvGA3ox2* expression recovered to the levels observed in air after 1 d. After transfer into air, *HvGA2ox3* expression was reduced about twofold but remained high (more than 100-fold compared with dry grains), while expression of *HvGA3ox2* was threefold less than before (Fig. 4A, B). *HvGA2ox1* and *HvGA2ox5* expression was similar in primary and secondary dormant grains after 1 d at 15 °C in air and reduced after 3 d of hypoxia treatment (Fig. S2A).

HvGA20ox1 expression was reduced by hypoxia after 1 d of treatment in comparison with air, but increased after and was surprisingly high during expression of secondary dormancy,

Table 2. Effect of fluridone (0.1 mM) applied during the induction of secondary dormancy treatment (15 °C, 5% O_2 for 3 d) or after the transfer in air on the subsequent germination at 15 °C in air. Results are shown as the mean of four replicates ± SD.

Pre-treatment for 3 d at 15 °C in 5% O_2 on:	Incubation medium after seed transfer at 15 °C in air	Germination (%) after 7 d
–	Water	98.7 ± 0.9
Water	Water	43.5 ± 7.5
Water	Fluridone	32.5 ± 5.0
Fluridone	Water	51.3 ± 13.3

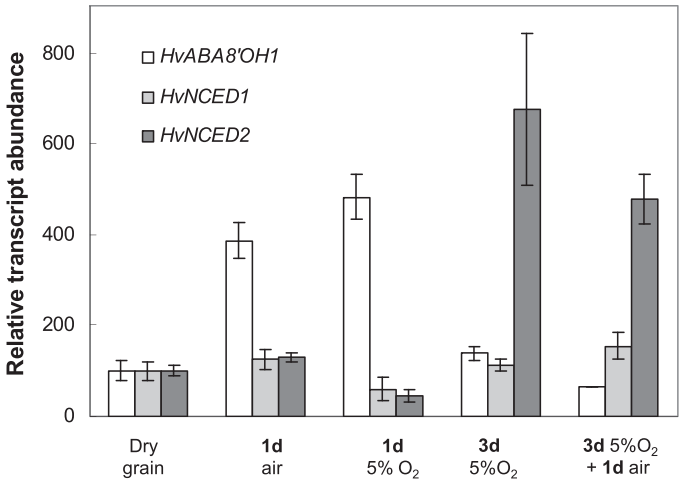

Fig. 3. Transcript abundance of *HvABA8'OH1* (ABA catabolism) and *HvNCED1* and *HvNCED2* (ABA synthesis) in embryos isolated from dormant grains before imbibition, after incubation at 15 °C for 1 d in air or in 5% O_2, for 3 d in 5% O_2, and for 1 d in air following the 3 d hypoxia treatment. Relative expression was calculated from real-time RT-PCR data from four reference genes, *HvActin*, *Hv18S*, *HvEF1α* and *HvMub1*, and was expressed in arbitrary units with a value of 100 assigned to the dry grains. Results are given as means of four replicates ± SD.

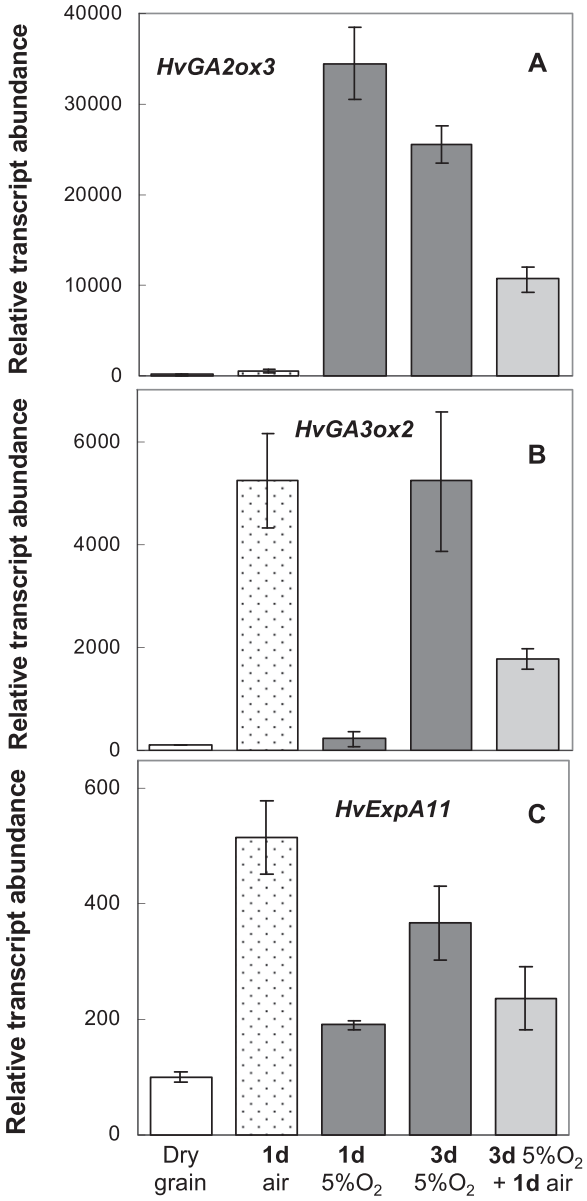

Fig. 4. Transcript abundance of*HvGA2ox3* (GA inactivation) and *HvGA3ox2* (GA synthesis) (A) and *HvExpA11* (GA response) (B) in embryos isolated from dormant grains before imbibition, after incubation at 15 °C for 1 d in air or in 5% O_2, for 3 d in 5% O_2, and for 1 d in air following the 3 d hypoxia treatment. Relative expression was calculated from real-time RT-PCR data from three reference genes, *Hv18S*, *HvEF1α*, and *HvMub1*, and was expressed in arbitrary units with a value of 100 assigned to the dry grains. Results are given as means of three replicates ± SD.

*i.*e. after transfer at 15 °C (Fig. S2B). *HvGA20ox3* expression was altered in the same way as *HvGA3ox2*, but with less amplitude (Fig. S2A). Analysis of the sensitivity to embryos from secondary and primary dormant grains to GA did not reveal any difference in GA sensitivity (data not shown). In barley, GA signalling induced expression of the expansin gene *HvExpA11* (Bahin *et al.*, 2011). After 1 d, its expression was strongly reduced in 5% O_2 in comparison with that in air. After 3 d in hypoxia, its expression increased but decreased again after transfer to air, i.e. in secondary dormant grains (Fig. 4C, light grey bar), being twofold less than in primary dormant grains imbibed at 15 °C in air (Fig. 4C, dotted bar).

Discussion

In barley, primary dormant grains cannot germinate at high temperature (Corbineau and Côme, 1996; Leymarie *et al.*, 2007) and this sensitivity to high temperature can be partly explained by a restriction of O_2 availability to the embryo (Lenoir *et al.*, 1986). The measurements of O_2 tension in embryos are in agreement with the previously estimated O_2 tensions at the level of the embryo (Edwards, 1973; Bradford *et al.*, 2008) and with the hypothesis of O_2 trapping at 30 °C by glumellae (Lenoir *et al.*, 1986). During barley caryopsis development, O_2 is produced by the pericarp layer, which contains chlorophyll (Borisjuk and Rolletschek, 2009), but at the end of maturation and dehydration, no more O_2 is produced and the internal parts of the grain are in hypoxia. As demonstrated previously (Corbineau and Côme, 1980; Lenoir *et al.*, 1986), primary dormant barley grains did not germinate properly in atmospheres containing less than 10% O_2 (Table S2). This sensitivity to hypoxia is modulated according to the dormancy state and temperature (Corbineau and Côme, 1980), and Bradford *et al.* (2008) demonstrated that embryos of after-ripened grains are less sensitive by one order of magnitude. It must be noted that the inhibition of germination by low O_2 availability at 30 °C (Table 1) is not related to an inability of the embryo to synthesise ATP (Côme *et al.*, 1988). Indeed, it has also been established by Al-Ani *et al.* (1985) that, in rice, wheat, and sorghum caryopses, the energy charge remained

close to 0.85, i.e. was similar to that measured in air, when the O_2 tension was reduced to 1–5%. The high energy charge value might be due to ATP production by fermentation (Al-Ani *et al.*, 1985). However, although P4Hs have been shown previously to be involved in the hypoxia response (Vlad *et al.*, 2007; Asif *et al.*, 2009), expression of the two P4Hs studied was not affected by the treatments. It cannot be excluded that other P4Hs could be implicated, as four other genes encoding predicted P4Hs exist in barley (*AK375293*, *AK365931*, *AK354544*, and *AK370642*). These genes can be classified into two groups according their sequence homologies in barley, and *AK250328* and *AK249666* were chosen as representative members of each group. However, this negative result is consistent with the hypothesis that the HIF system does not exist in plants for hypoxia sensing and with the new findings on low O_2 sensing by plant-specific group VII ethylene-response factor transcription factors (Bailey-Serres *et al.*, 2012).

The results presented here showed that secondary dormancy can be induced by hypoxia at low temperature in barley (Fig. 1), as already shown in other species (Côme and Tissaoui, 1968; Esashi *et al.*, 1978; Lonchamp and Gora, 1979; Pekrun *et al.*, 1997). This induction of secondary dormancy could occur in soils where the O_2 level usually does not fall below 19% (Richard and Boiffin, 1990), but can decrease to 1% or even less in soils that are maintained at field capacity or are flooded (Gambrell *et al.*, 1991). The secondary dormancy induced by 3 d in hypoxia (5% O_2 at 15 °C) was similar to that induced by 3 d at 30 °C (i.e. when the embryo was in hypoxia under the grain envelopes), as only 30% of the grain population could subsequently germinate within 7 d at 15 °C in air (Fig. 1; Leymarie *et al.*, 2008; Hoang *et al.*, 2012). However, the mechanisms involved in both processes are different.

The O_2 availability to the embryo would have additional effects on synthesis of, and sensitivity to, ABA and GA, altering the balance to either promote or delay germination (Benech-Arnold *et al.*, 2006; Bradford *et al.*, 2008). The embryo sensitivity to ABA of secondary dormant grains was reported here to be similar to that of embryos isolated from primary dormant grains (Fig. 2B). In contrast, embryos isolated from secondary dormant grains obtained by a 30 °C treatment are more sensitive to ABA (Leymarie *et al.*, 2008). The ABA content observed after 1 d at 15 °C in air was reduced twofold when compared with that of dry grains, and this reduction was associated with increased expression of the *HvABA8'OH1* gene. The embryo ABA content decreased more slowly in hypoxia, but the *HvABA8'OH1* transcript abundance was higher in hypoxia than in air after 1 d. Such induction of *HvABA8'OH1* by hypoxia has been observed previously in barley after 14 h of imbibition at 30 °C (Mendiondo *et al.*, 2010). As the 1 d hypoxia treatment did not induce *HvNCED* transcripts, the maintenance of high ABA content could be then related to post-transcriptional mechanisms. Nevertheless, an alteration of the ABA-8'-hydroxylase activity by hypoxia could not be ruled out as this enzyme is a monooxygenase (Krochko *et al.*, 1998) with a putative feedback regulation of gene transcription. When the hypoxia treatment was extended up to 3 d, the changes in ABA content were slightly different according to the type of pre-treatment. Indeed, it decreased more in grains incubated at 30 °C than in those placed in hypoxia (Hoang *et al.*, 2012; Fig. 2A), but the *HvABA8'OH1* level was similar (Figs 2A and 3; Hoang *et al.*, 2012). *HvABA8'OH1* transcript expression appeared to be less regulated by hypoxia than by development or other environmental factors, as shown previously (Chono *et al.*, 2006; Millar *et al.*, 2006; Gubler *et al.*, 2008; Leymarie *et al.*, 2008; Hoang *et al.*, 2012). The major difference between the inductive treatments appeared after the transfer to 15 °C in air, as the ABA content was high in the case of the 30 °C treatment (2.5 pmol mg^{-1} DW; Hoang *et al.*, 2012), while it decreased down to 1.32 pmol mg^{-1} DW in the case of the hypoxia treatment (Fig. 2A). The difference in ABA content observed between the two inductive treatments appeared to be related to differential *HvNCED* expression. The *HvNCED1* gene was more highly expressed in grains treated at 30 °C (Hoang *et al.*, 2012), while *HvNCED2* (Figs 2A and 3) expression was higher in the hypoxia-treated grains but appeared to have a smaller effect on overall ABA content. This inductive effect of hypoxia treatment on *HvNCED2* was late, as it was not revealed after 1 d. Fluridone application had no effect on hypoxia-induced secondary dormancy (Table 2) but inhibited the induction and expression of secondary dormancy by high temperature (Leymarie *et al.*, 2008). All these data suggest that induction of secondary dormancy by hypoxia is less regulated by ABA than induction by high temperature. With regard to GA metabolism, the hypoxia treatment induced the main changes in the expression of key genes: *HvGA2ox3* was induced 64-fold, while *HvGA3ox2* was repressed 16-fold (Fig. 4) compared with expression observed in air after 1 d. The GA signalling pathway evaluated by the level of *HvExpA11* expressed was also repressed during this period. After 3 d in hypoxia, the expression of *HvGA3ox2* and *HvExpA11* tended to recover to that observed in air after 1 d, showing the importance of GA metabolism regulation in the first steps of the hypoxia response and the induction of secondary dormancy. The ratio between the most highly expressed genes, *HvGA3ox2*/*HvGA2ox3*, was 9 for primary dormant grains at 15 °C while it was between 0.16 and 0.32 for secondary dormant ones. This suggested a reversal in the GA metabolism from synthesis in dormancy release in primary dormant grains to increased catabolism in the induction of secondary dormancy that is observed by 1 d of hypoxia. More precisely, the incubation in hypoxia at 15 °C was related mainly to *HvGA2ox3* (after 1 and 3 d) and *HvGA3ox2*, *HvGA20ox3* and *HvGA20ox1* (after 1 d) (Figs 4A and S2), while high temperature enhanced mainly *HvGA2ox1*, *HvGA2ox5*, and, to a lesser extent, *HvGA2ox3* expression (Hoang *et al.*, 2012). The induction of *HvGA2ox3* by hypoxia persisted after the transfer to air, which was not observed in the case of high-temperature induction. The high expression level of *HvGA20ox1* after the transfer was surprising, as it was not consistent with the dormancy state (Fig. S2B). The 30 °C treatment was related mainly to low expression of *HvGA3ox2* during the treatment and after the transfer, while *HvGA20ox3* was surprisingly induced (Hoang *et al.*, 2012). All these data show that induction of secondary dormancy induced GA

inactivation and reduced GA synthesis, presumably leading to reduced active GA content. Moreover, the expression of *HvExpA11* that is related to activation of GA signalling (Yamauchi *et al*., 2004; Bahin *et al*., 2011), and then to GA content, was in accordance with the dormancy state, as its expression decreased early during the hypoxia treatment and after the transfer (Fig. 4B). The regulation of gene expression was, however, different according to the type of induction of secondary dormancy, with *HvGA2ox3*, *HvGA3ox2*, and *HvGA20ox3* (Figs 4 and S2) appearing to have a major role in the case of hypoxia induction, while *HvGA2ox1*, *HvGA2ox3*, *HvGA2ox5*, and *HvGA3ox2* are involved mainly at high temperature (Hoang *et al*., 2012).

The results presented here show that the induction of secondary dormancy in barley grains by hypoxia is regulated differently from that induced by high temperatures (Hoang *et al*., 2012). The induction of secondary dormancy by hypoxia appears to be more regulated by GA and less by ABA than the induction by high temperature. Although high temperature has an indirect effect on O_2 availability, these two factors must have then specific signalling pathways.

Supplementary data

Supplementary data are available at *JXB* online.

Supplemental Table S1. Oligonucleotide sequences of primers used for RT-PCR experiments.

Supplemental Table S2. Germination percentages at 15 °C at various O_2 tensions.

Supplemental Fig. S1. Relative transcript abundance of the P4H genes *HvP4H1* and *HvP4H2*.

Supplemental Fig. S2. Relative transcript abundance of *HvGA2ox1*, *HvGA2ox5*, *HvGA20ox1*, and *HvGA20ox3*.

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